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A novel glutathione modified chitosan conjugate for efficient gene delivery

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ABSTRACT

A novel non-viral gene vector based on poly[poly(ethylene glycol) methacrylate] (PMPEG) and L-glutathione (GSH) grafted chitosan (CS) has been fabricated. First, well-defined brush-like PMPEG living polymers with dithioester residues were prepared by the reversible addition–fragmentation chain transfer (RAFT) polymerization and grafted onto the allylchitosan via radical coupling method. Then, the tripeptide GSH was introduced onto the end of PMPEG chain to give a CS–PMPEG–GSH conjugate. In comparison with pristine chitosan, CS–PMPEG–GSH conjugate could not only condense plasmid DNA (pDNA) and prevent the condensed CS–PMPEG–GSH/pDNA nanoparticle self-aggregation, but also increase the binding ability to cell membrane efficiently and improve decondensed ability of pDNA from the nanoparticles in cytoplasm which thus has resulted in the higher transfection efficiency in mouse embryonic fibroblast cells (NIH3T3). In addition, cytotoxicity assays showed that the conjugate is less cytotoxic than CS, and still retain the cationic polyelectrolyte characteristic as chitosan. These results indicate that the non-viral vector is a promising candidate for gene therapy in clinical application.

1. Introduction

Gene therapy has gained more and more attention over the past two decades for treatment of diverse human diseases [1–3]. An efficient and safe delivery system that delivers the therapeutic genes to a specific target tissue or organ is the prerequisite for successful gene therapy [4,5]. Currently, gene delivery system is mainly categorized to be viral and non-viral vectors. Viral vectors are biological systems derived from naturally evolved viruses capable of transferring their genetic materials into the host cells, thus are very effective in achieving high efficiency for both gene delivery and expression. However, the limitations associated with viral vectors, in terms of safety, immunogenicity, low transgenic size and high cost, have encouraged researchers to focus on non-viral vectors [6–8]. Cationic lipids, synthetic and natural polymers consist of the main non-viral vectors. Although cationic lipids are easily transferred into cell membranes, they could be rapidly cleared from the bloodstream, widely distributed in the body and relatively large in size, which limit their applications [9–11]. In comparison, cationic polymers, especially natural cationic polymers, such as chitosan etc., which are biocompatible, biodegradable and low toxic, as alternative, have been widely used as gene vectors and much attention has been paid on [12,13].

Chitosan is composed of D-glucosamine and *N*-acetyl-D-glucosamine linked by β (1, 4)-glycosidic linkages. Besides good biocompatibility, biodegradability, low toxicity and multiple functionalizations, the positive characteristics make chitosan a good gene carrier candidate and series of experiments confirmed that chitosan could effectively combine with pDNA and protect it from nuclease degradation. In addition, amino group of chitosans could bind with the negatively charged cell membrane surface by electrostatic attraction and hydrogen bond, which could increase cell uptake of pDNA [14]. Previous studies have shown that the binding affinity of chitosan for pDNA, the stability and the transfection efficiency of the chitosan/pDNA complexes are dependent on several formulation parameters, such as the molecular weight (Mw) of chitosan, degree of deacetylation (DD), stoichiometry of the chitosan/DNA complex (N/P ratio, charge ratio of amine (chitosan) to phosphate (DNA)), plasmid concentration, serum concentration, pH of the transfection medium, cell type and so on [15–21]. However, the specificity and transfection efficiency of chitosan are usually very low, which need to be overcome before its use in clinical trials. In order to circumvent the disadvantages mentioned above and further improve gene transfection, many efforts have been done and various modifications have been carried out on chitosan. For example, in order to enhance the circulation time of chitosan/DNA nanoparticle in bloodstream, hydrophilic PEG was grafted to chitosan, which could not only reduce the plasma clearance by lung endothelia uptake, but

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also protect DNA degradation from plasma nucleases [22–24]. In order to modulate hydrophobic complex interaction with cells and assist dissociation of polymer/DNA complexes, chitosan was modified with deoxycholic acid to give a conjugate, which showed superior gene condensation than unmodified chitosan. Inefficient release of the polymer/DNA complexes from endocytic vesicles into cytoplasm is one of the primary causes of poor gene delivery [25]. Kim et al. [26] modified chitosan with a urocanic acid bearing an imidazole ring, which can play the crucial role in endosomal rupture through a proton sponge mechanism. In addition, various specific ligands such as galactose [27–29], transferring [30], folate [31].and mannose [32] were also incorporated into chitosan for cell specificity. As an alternative, recently thiolated chitosan has been designed and used as gene vectors. The free sulphydryl groups on its side chains can form disulfide with mucin glycoproteins on cell membranes, thereby promoting cellular uptake of the thiolated chitosan/pDNA complexes [33–37]. However, most of the modifications above mentioned were via amidation reaction which could consume the amino group of chitosans and influence the cationic property of CS-mediated gene delivery, even resulted in higher cytotoxity.

Glutathione in its reduced form (GSH), a small tripeptide formed by glutamic acid, cysteine and glycine, widely presenting in a variety of animal tissues, can role as specific peptide ligand to promote cell adhesion. Meanwhile, free thiol group is able to chelate divalent metal ions, which are essential to DNase for its activity [38]. Permeation studies carried out with model drugs across intestinal mucosa demonstrated that the combination of thiolated polymers with reduced glutathione as low molecular mass permeation mediator led to a significantly improved permeation-enhancing effect of thiomers [39,40]. Chitosan–glutathione conjugate which synthesized by combining free GSH on chitosan directly exhibited improved mucoadhesive and cohesive properties, and the highest permeation-enhancing effect among other thiomers [41]. Based on previous studies about the structure and application of GSH, a novel chitosan–glutathione conjugate has been designed in our lab which might lead to a new generation of thiomers for gene vectors.

In this work, by employing the reversible addition–fragmentation chain transfer method, a series of well-defined PEG brush-like PMPEG living chains with dithioester residues was prepared, which then was grafted onto the allylchitosan via the radical coupling method [42]. Next, GSH was further introduced to the CS by amidation reaction between the carboxyl group of dithioester residue and the amino group of GSH. Based on the above idea we have synthesized the CS–PMPEG–GSH conjugate. Then CS–PMPEG–GSH/pDNA complexes were prepared and characterized in terms of size, zeta potential, pDNA condensation efficiency and enzymatic stability. In addition, the toxicity and transfection efficiencies of CS–PMPEG–GSH/pDNA have been monitored, and a detailed mechanistic assessment was performed on the transfection process, including monitoring of cellular uptake and GSH responsive intracellular pDNA release.

2. Experimental

2.1. Materials

Chitosan was purchased from Gold-Shell Biochemical Co., Ltd. (Mw = 50 kDa, degree of deacetylation (DD) = 90%, Zhejiang, China). Allyl bromide, analytical grade, was obtained from Xingjing Chemical Company (Beijing, China). Poly(ethylene glycol) methacrylate (MPEG, Mn = 526, Aldrich) was purified by basic aluminum oxide column. 4,4′-Azobis(4-cyanopentanoic acid) (ACVA, Fluka, 98%) was recrystallized from methanol. (4-Cyanopentanoic acid) dithiobenzoate (CPADB) as chain transfer agent (CTA) was prepared by using a method as previous literature [43] (^{1}H NMR ($CDCl_3$,δ): aromatic 7.4–8.0 ppm, —C**H**$_2$—C**H**$_2$— 2.5–3.0 ppm, —C**H**$_3$ 2.0 ppm, yield is about 20%). L-glutathione (GSH, reduced form, 98%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 98.5%), and N-hydroxysuccinimide (NHS, AR) were obtained from Aladdin (Shanghai, China). Trypsin-EDTA, agarose, Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumerma bromide (MTT) were obtained from DingGuo Biotech. Co. Ltd (Tianjin China). Branched polyethylenimine (PEI, 25 kDa) were purchased from Sigma (St. Louis, MO). The plasmid pEGFP-N1 (4.7 kb; Clontech, Palo Alto, CA, USA) encoding enhanced green fluorescent protein (EGFP) is driven by immediate early promoter of CMV. The plasmid DNA (pDNA) was maintained and propagated in DH5α strain of *E. coli*. The plasmids were purified by use of the Endfree plasmid kit (Tiangen, China), and purity and concentration of plasmids were confirmed by spectrophotometry (A260/A280).

2.2. Characterization instruments

^{1}H NMR spectra were recorded on a Varian UNITY-plus 400 spectrometer operated at 400 MHz with the internal solvent peak as a reference.

IR spectra were recorded with a Bio-Rad FTS 135 Fourier transform infrared (FTIR) spectrometer in the range of 3500–500 cm^{-1} using KBr pellets.

The molecular weights and polydispersities (PDI) of PMPEG were determined with size exclusion chromatography ( ) equipped with Waters Shodex KW-804 columns. The SEC parameters were: 25 °C, 0.2 M $NaNO_3$ as mobile phase, 0.6 mL/min.

The particle size and morphology of CS–PMPEG–GSH/pDNA complexes were determined by a multimode IIIa AFM (Veeco Metrology, USA) under ambient conditions, and samples were prepared by dropping the solution on mica.

The surface charge of CS–PMPEG–GSH/pDNA complexes was assessed by laser light scattering using a zeta potential analyzer (Zetasizer 3000HS, Brookhaven). Determination of zeta potential was done with a dilution of 1:10 in sodium acetate buffer and the measurement was performed at 25 °C with a scattering angle of 90° and an electric field strength of 7 V/cm.

2.3. Cell culture

The cell line mouse embryonic fibroblast cells (NIH3T3) were purchased from ATCC (Teddington, UK). NIH3T3 was maintained in DMEM containing 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were maintained at 37 °C in a humidified and 5% CO_2 incubator.

2.4. Synthesis

2.4.1. Preparation of PMPEG living polymer

A typical polymerization was described as follows: MPEG (2.104 g, 4.0 mmol), ACVA (5.6 mg, 0.020 mmol), and chain transfer agent CPADB (27.9 mg, 0.10 mmol) were dissolved in methanol (10 mL) and degassed by three freeze–pump–thaw cycles. Polymerization was conducted in water bath at 60 °C for 24 h and stopped by quenching the reaction flask in ice water. After concentrated and precipitated in cold diethyl ether, PMPEG, a viscous and pink polymer, was collected and dried under vacuum.

2.4.2. Allylation of chitosan

Allyl–chitosan was synthesized according to a previous report [44,45]. Chitosan (10.0 g) was dissolved in 75 mL of deionized water together with 2.5 g NaOH and 0.1 g sodium borohydride. Then 10 mL of allyl bromide was added at 40 °C. After that, the mixture was stirred for 3 h at 60 °C and neutralized with acetic acid. The obtained product was further purified by repeated precipitations in excessive ethanol, dried to a constant weight under vacuum and collected as pale yellow powder. The allylation ratio was 15% determined by ^{1}H NMR.